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14. ABSTRACT
Docetaxel (DTX), a semi-synthetic analog of paclitaxel, has emerged as the standard of care for chemotherapy of hormone-resistant prostate cancer (PCa). However, most patients treated with DTX ultimately develop resistance to the drug and succumb to the disease. Therefore, understanding the mechanisms underlying DTX resistance is a priority area in PCa research. Increasingly, it is being suggested that cancer cells may use common pathways for disease aggressiveness/metastatic spread and chemotherapeutic resistance. Therefore, we investigated the role of CXCL12/CXCR4 signaling, which is known to promote invasion and metastasis, in DTX-resistance of PCa cells. Our data demonstrated that CXCL12 treatment rescued the PCa cells from DTX-induced cytotoxicity and G2/M mitotic arrest. Furthermore, the cytoprotective effect of CXCL12 was abolished upon pretreatment of PCa cells with AMD3100 (a small molecule antagonist of CXCR4) or siRNA-mediated silencing of CXCR4, thus confirming the role of CXCR4 in DTX-resistance. Immunofluorescence analyses revealed enhanced polymerization of microtubules in DTX-treated PCa cells. In accordance with this, immunoblot analyses demonstrated increased levels of detyrosinated (glu-) and acetylated (ace-) tubulins, specific markers of the polymerized tubulin, in PCa cells treated with DTX. Co-treatment of CXCL12 abrogated DTX-induced stabilization of microtubules in the PCa cells, an effect that was diminished when the cells were pre-treated with AMD3100. Data from our mechanistic studies suggest the role of PAK4-dependent LIMK1 activation in the CXCL12-induced resistance to docetaxel toxicity and microtubule destabilization. Altogether, our findings demonstrate the role of CXCL12/CXCR4 signaling axis in DTX-resistance of PCa cells and thus could be an attractive target for therapeutic enhancement of DTX.

15. SUBJECT TERMS
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INTRODUCTION

Docetaxel (DTX), a semi-synthetic analog of paclitaxel, has emerged as the standard of care for chemotherapy of hormone-resistant prostate cancer. Docetaxel confers its anti-neoplastic activity by inhibiting microtubule depolymerization, which leads to G2/M mitotic arrest and subsequent apoptosis [1]. However, most patients treated with DTX ultimately develop resistance to the drug and succumb to the disease [2]. Therefore, understanding the mechanisms underlying DTX resistance is a priority area in prostate cancer research. Previously it has been reported that CXCL12/CXCR4 signaling play an important role in microtubule organization in immune cells [3] and induction of mitotic catastrophe (G2/M arrest) upon inhibition of this signaling pathway in ovarian cancer cells [4]. Thus, based on these studies, we **hypothesize** that CXCL12-CXCR4 signaling axis promotes docetaxel resistance by counteracting the microtubule stabilizing action of docetaxel.

To test our hypothesis, we have proposed two specific aims:

1) To investigate the role of CXCL12/CXCR4 signaling in microtubule dynamics of prostate cancer cells.
2) To examine if activation of this signaling node restricts docetaxel-induced microtubule stability and toxicity in prostate cancer cells *in vitro*.

We expect that the proposed investigations will provide a strong rationale for CXCL12/CXCR4 targeting to overcome docetaxel resistance and improve therapeutic outcome in prostate cancer.

BODY

Task 1: To examine the effect of CXCL12-CXCR4 signaling on microtubule dynamics in prostate cancer cells.

CXCR4 is overexpressed in prostate cancer cells: First we examined the expression level of CXCR4 and CXCL12, a sole ligand of CXCR4 in four prostate cancer (LNCaP, C4-2, PC3 and DU145) cells at transcription and protein level, by quantitative RT-PCR and immunoblot/ELISA assays, respectively. In addition we also examined their expression in two normal prostate epithelial (RWPE1 and RWPE2) cells. Our

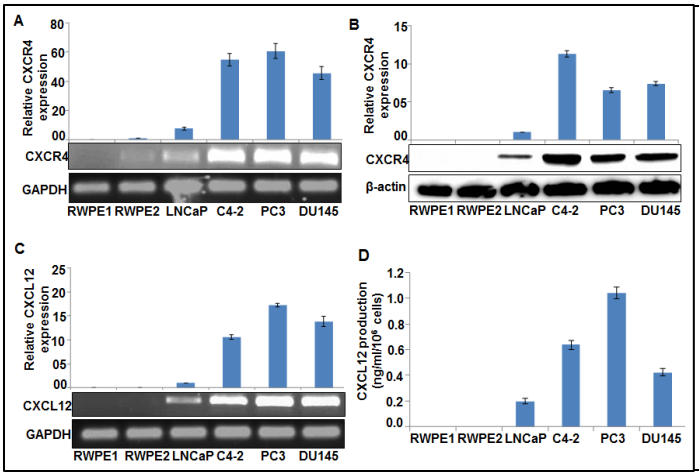

Figure 1: CXCL12 and CXCR4 expression in prostate cancer cells. Expression of CXCR4 (A & B) and CXCL12 (C & D) was examined at mRNA and protein level. Data (mean ± SD, n=3) show an aberrant expression of CXCR4 and very low levels of CXCL12 in all PCa cells whereas, no or negligible expression of CXCR4 as well as CXCL12 was observed in normal prostate epithelial cells.

data demonstrate an aberrant expression of CXCR4 and very low levels of CXCL12 (0.2 -1.1 ng/mL/10^6 cells) in all the prostate cancer cell lines (LNCaP, C4-2, DU145 and PC3). Whereas, no or negligible expression of both CXCR4 **(Figure 1 A and B)** and CXCL12 **(Figure 1 C and D**) was noted in prostate epithelial cell lines (RWPE1 and RWPE2) **(Figure 1)**. Taken together, our data show that CXCR4 is overexpressed in prostate cancer cells.

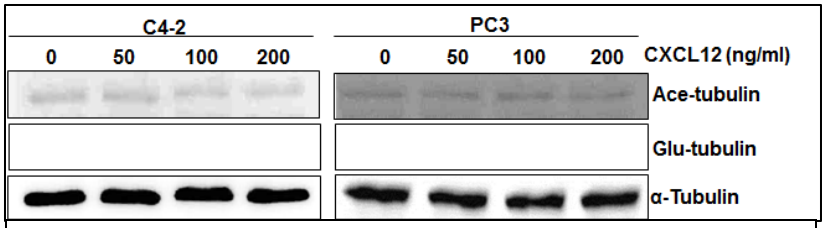

Figure 2: Effect of CXCL12 on the acetylated (Ace), detyrosinated (Glu) and total α-tubulin. Prostate cancer cells were treated with the different doses of CXCL12 for 24h. After treatment immunoblot analysis was performed to examine the expression on Ace, Glu and total tubulin. Data show that there is no basal expression of Glu-tubulin in both the cell lines, whereas no significant change was observed in the basal expression of Ace, and total tubulin upon CXCL12 stimulation.

Basal expression of acetylated (Ace) tubulin is unchanged after CXCL12 treatment. To investigate the effect of CXCL12 treatment on prostate cancer (C4-2 and PC3) cells, we treated cells with various doses of CXCL12 (0-200 ng per mL) for 24h and post-treatment morphological changes was examined by phase contrast microscope. No significant changes were observed in the morphology of both the prostate cancer (C4-2 and PC3) cells after CXCL12 treatment as compared to untreated cells. Next, we examined the effect of CXCL12 on the expression of acetylated (Ace), detyrosinated (Glu) by immunoblot analysis. Data demonstrate that C4-2 and PC3 cells do not exhibit the expression of Glu-tubulin; whereas very low basal expression of Ace-tubulin in both the cell lines was seen. Furthermore, we observed that expression of Ace-tubulin and total tubulin is remained unchanged following treatment of CXCL12 **(Figure 2)**.

<u>Task 2: To investigate the effect of CXCR4 activation on docetaxel-induced microtubule sensitivity and growth suppression.</u>

CXCL12/CXCR4 signaling protects prostate cancer cells from DTX-induced cytotoxicity: C4-2 and PC3 cells were grown in 96 well plate and treated with different doses of DTX (0-30 nM) in presence or absence of CXCL12 (100 ng/ml). After 48 h of treatment viability of cells was evaluated by WST1 assay and IC_{50} value was calculated. Our data demonstrate that CXCL12 treatment induces resistance to DTX-induced cytotoxicity in C4-2 and PC3 cells **(Figure 3)**. CXCL12-induced chemoresistance effect was clearly evident by the increase in IC_{50} values of DTX in CXCL12-treated C4-2 and PC3 (> 30.0 nM) cells as compared

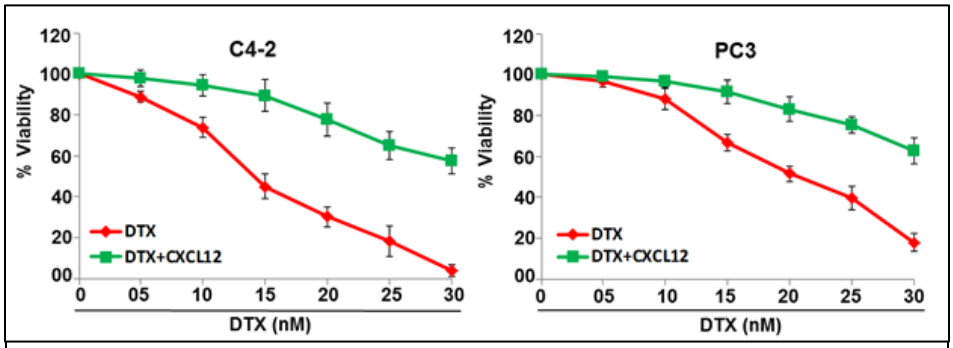

Figure 3: CXCL12 rescue the docetaxel-induced cytotoxicity: C4-2 and PC3 cells were treated with various doses of docetaxel (DTX; 0–30 nM) in the presence and absence of CXCL12 (100 ng/mL). After 48 h, viability of cells was examined WST-1 assay. Data are presented as percent viability with respect to untreated or CXCL12 only-treated cells.

to control cells (~13.5 and 19.0 in C4-2 and PC3, respectively). To ascertain the involvement of CXCR4 in the CXCL12-mediated rescue effect, we silenced CXCR4 expression using specific siRNAs prior to the CXCL12 and DTX treatment and effect on cell viability was examined. Data show that CXCR4-targeted siRNAs led to effective silencing of CXCR4 **(Figure 4)**. Furthermore, we observed that CXCL12-induced chemoprotective effect is abolished in cells transfected with CXCR4-targeted siRNAs **(Figure 5A)**. Moreover, PCa cells were treated with AMD3100, a CXCR4 antagonist, prior to the CXCL12 and DTX treatment. Our data show that pre-treatment of PCa cells with CXCR4 antagonist (AMD3100) abolished CXCL12-induced DTX resistance in PCa cells **(Figure 5B)**. Together, these data demonstrate that CXCR4 mediates CXCL12-induced chemoresistance in PCa cells.

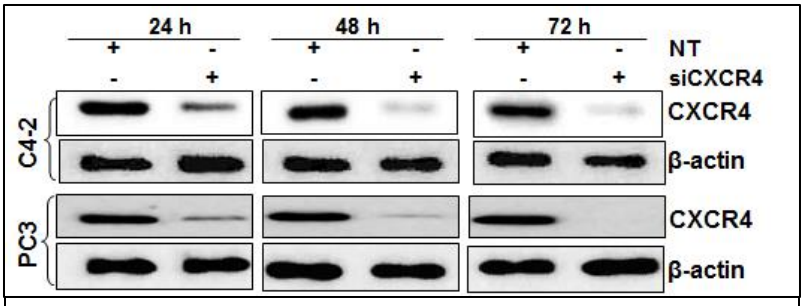

Figure 4: Silencing of CXCR4 through specific siRNAs. C4-2 and PC3 cells were grown in 6-well plate and transiently transfected with non-target (NT), CXCR4-targeted siRNAs for 24-72h. After treatment, total protein was isolated and subjected to immunoblot analysis to assess the CXCR4 expression. β-actin was used as loading control.

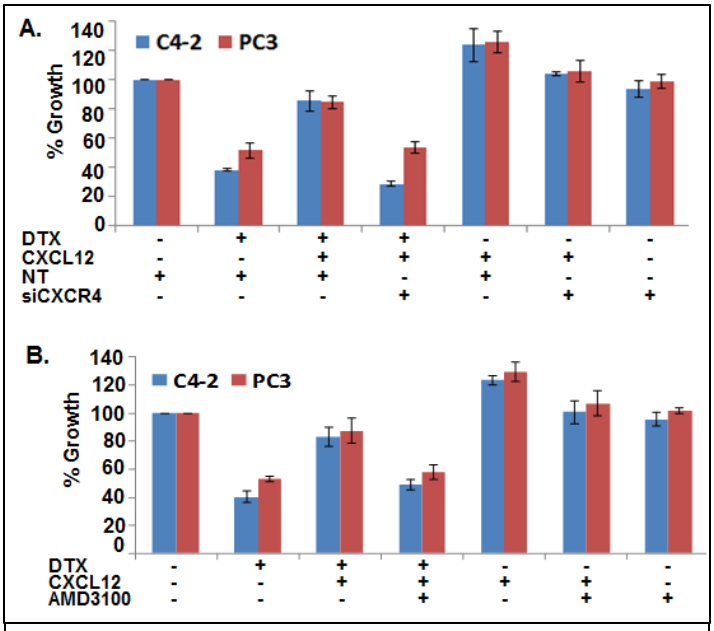

Figure 5: CXCR4-mediates the rescue effects of CXCL12 on docetaxel-induced cytotoxicity. A. C4-2 and PC3 cells were grown in 96 well plate. Thereafter, cells were treated with either CXCR4 targeting siRNAs or non-targeting control (NT-siRNA). After 24 h of transfection cells were treated with DTX (20 nM) in absence or presence of CXCL12 (100 ng/mL) and growth was monitored by WST-1 assay after 48h. **B.** C4-2 and PC3 cells were treated with the AMD3100 (5 µg/mL), a CXCR4 antagonist, 1h prior to the treatment of CXCL12 followed by DTX treatment. After 48 h of treatment, viability of cells was examined by WST-1 assay. Data is presented as mean± S.D.

CXCL12 treatment relieves the docetaxel-induced mitotic arrest. Several lines of evidence suggest that DTX causes cytotoxicity by causing G2/M phase cell cycle arrest [5, 6]. Thus, next we examined whether CXCL12/CXCR4 has any effect on the DTX-induced G2/M phase cell cycle arrest. For this, C4-2 and PC3 cells were treated with DTX alone or in presence of AMD3100 and/or CXCL12 pre-treatment, and fraction of cells in different phase of cell cycle was analyzed by flow cytometry assay. Similar to previous report [6], we also observed the arrest of cells in G2/M phase of cell cycle upon DTX treatment, which is clearly evident by higher number (62.09% and 61.6% in C4-2 and PC3, respectively) of cells to be arrested in

G2/M phase in DTX-treated cells as compared to control C4-2 (11.52%) and PC3 (16.0%) cells **(Figure 6)**. Interestingly, our data demonstrate that CXCL12 treatment rescued the PCa cells from DTX-induced G2/M mitotic arrest, and this effect was abolished upon pretreatment of PCa cancer cells with AMD3100 (Figure 2). Altogether, our results suggest that CXCL12/CXCR4 relieves DTX-induced G2/M phase cell cycle arrest in PCa cells and, thus may protect them from the cytotoxic effect of DTX.

CXCL12/CXCR4 signaling counteracts docetaxel-induced microtubules stabilization. DTX is a microtubule-stabilizing agent, which causes mitotic arrest following binding to the polymerized tubulins and subsequent blockage of their depolymerization [5, 7]. Therefore, we sought to investigate if CXCL12/CXCR4 signaling counteracts the effect of DTX on the stabilization of microtubules. In this direction, stabilization status of microtubule in PCa cells treated with DTX alone or in the presence of CXCL12 was examined by immunofluorescence assay using a specific marker of polymerized tubulin (glu-tubulin) [5, 8]. Our data demonstrate enhanced polymerization of microtubules in DTX-treated PCa cells **(Figure 7A, next page)**. Notably, treatment of CXCL12 abrogated DTX-induced stabilization of microtubules in the PCa cells, an effect that was reversed following inhibition of CXCR4 by AMD3100 **(Figure 7A, next page)**. Further to confirm these findings, the expression of glu-tubulin and acetylated (ace-) tubulin, specific markers of polymerized tubulin [5, 8], was determined by immunoblot assay. In accordance to the immunofluorescence study, our immunoblot analysis also suggests the counteracting role of CXCL12/CXCR4 signaling on the DTX-induced stabilization of microtubules **(Figure 7B, next page)**. Taken together, these findings demonstrate that the activation of CXCL12/CXCR4 signaling rescue the PCa cells from DTX-induced G2/M phase cells cycle arrest and subsequent cell death by counteracting the DTX-induced microtubule stabilization.

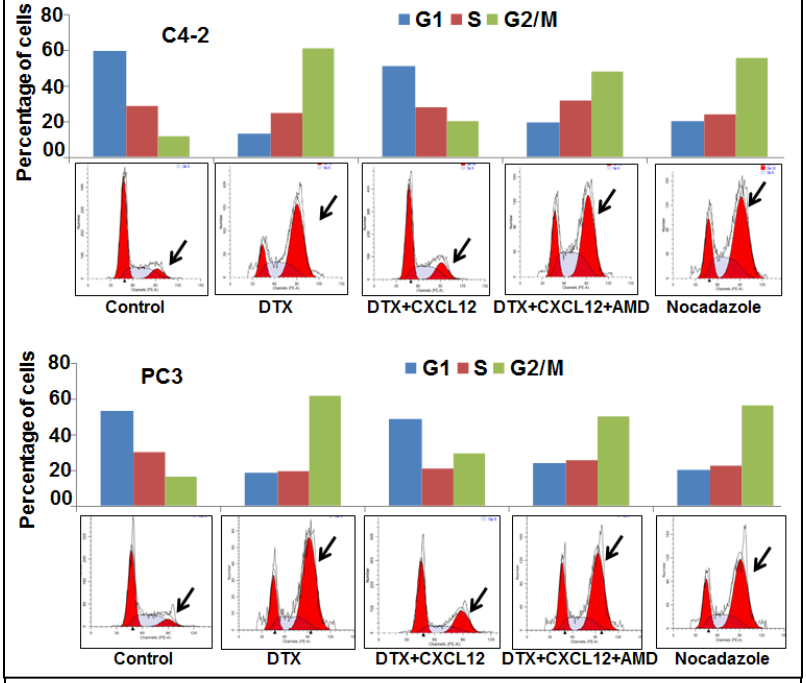

Figure 6: Activation of CXCL12/CXCR4 signaling relieves docetaxel-induced G2/M cell cycle arrest. Synchronized C4-2 and PC3 cells were treated with docetaxel (DTX; 20nM) alone or in combination with AMD3100 (5 µg/mL) and/or CXCL12 (100 ng/mL). After 24h of treatment cells were fixed, stained with propidium iodide and analyzed using flow cytometry. Data show a G2/M phase-arrest in DTX-treated cells. CXCL12 abrogated DTX-induced G2/M arrest, which was reversed in the cells pre-treated with AMD3100. Nocadazole was used as positive control.

Inhibition of LIMK1 abrogates CXCL12-induced resistance to docetaxel toxicity and microtubule destabilization. Next, we explored the possible involvement of LIMK1 (an important downstream molecule of CXCL12/CXCR4 signaling [9] and known to regulate the stability of microtubules [10]), in the rescue effects mediated by CXCL12/CXCR4. Our data show an increase in the phosphorylation of LIMK1 following CXCL12 stimulation in PCa cells **(Figure 8A)**. Phosphorylation of LIMK1 in CXCL12 treated cells increases in time dependent manner, which starts within 10 min and sustains upto 1 h **(Figure 8A)**. Moreover, we observe that CXCL12-induced LIMK1 phosphorylation is abrogated following pretreatment of AMD3100, thus, suggesting the role of CXCL12/CXCR4 signaling in the activation of LIMK1 **(Figure 8B)**. Next, PCa cells were treated with LIMKi3, a LIMK1 inhibitor; prior to the treatment of CXCL12 and DTX and effect on DTX-induced cytotoxicity was determined. Our data demonstrate that rescue effect of CXCL12/CXCR4 signaling from DTX-induced cytotoxicity is abolished upon LIMK1 inhibition in both the PCa cell

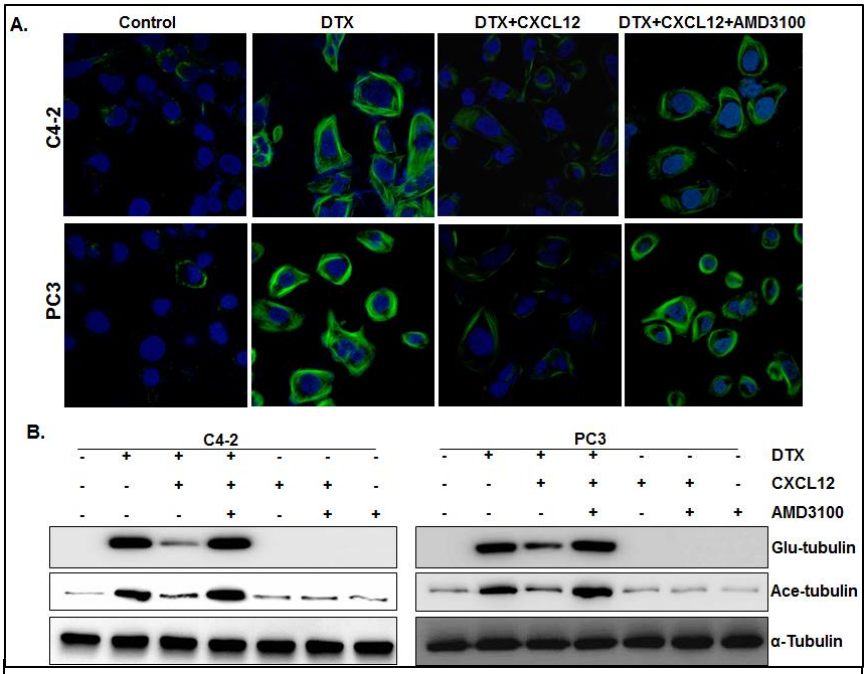

Figure 7: Effect of CXCL12/CXCR4 signaling on the docetaxel-induced microtubules stabilization. A. PCa cells (C4-2 and PC-3) were grown on glass bottom plate and treated with DTX alone or in combination with pre-treatment of AMD3100 (5 µg/mL) and/or CXCL12 (100 ng/mL). After the treatment cells were fixed, stained using glu-tubulin and examined under confocal microscope. **B.** Total protein was collected from the PCa cells treated with DTX alone or in combination with pre-treatment of AMD3100 and/or CXCL12 for 24 h. Thereafter, expression of detyrosinated (Glu), acetylated (Ace) and total α-tubulin was examined by immunoblot analysis. Data show increased stabilization of microtubules in the cells treated with DTX alone. Whereas, CXCL12 abrogated DTX-induced microtubule stabilization, which was diminished in the cells pre-treated with AMD3100.

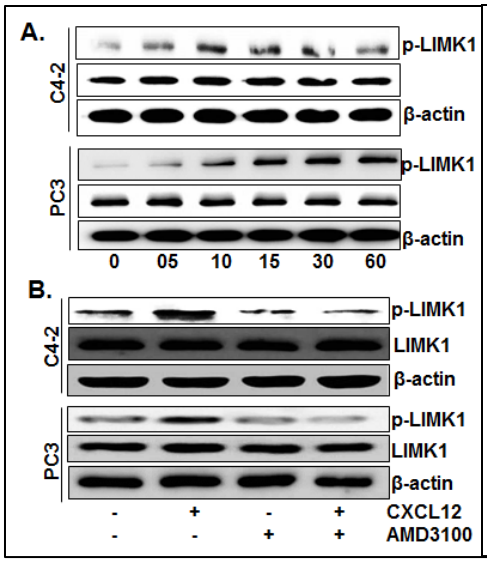

Figure 8: CXCL12 activates LIMK1 through CXCR4. A. Cells were grown in 6-well plate and treated with CXCL12 (100 ng/mL) for various time intervals (0-120 min). Post treatment total protein was collected and expression of p-LIMK1 and total-LIMK1 was determined by immunoblot analysis. **B.** Cells were pretreated with AMD3100 (5 µg/mL) 1h prior to the CXCL12 treatment (100 ng/mL). After 60 min, effect on the expression of p-LIMK1 and total-LIMK1 was examined by immunoblot assay. β-actin was used as an internal control.

lines **(Figure 9)**. Further examination revealed an enhanced microtubule stabilization upon inhibition of LIMK1 while, co-treatment of LIMKi3 along with DTX has more potent effect on microtubule stabilization in PCa cells **(Figure 10)**. Interestingly, the inhibition of LIMK1 abolishes the counteracting effect of CXCL12/CXCR4 signaling on the DTX-induced microtubule stabilization **(Figure 10)**. It has been suggested that several proteins referred as microtubule associated proteins (MAPs), bind to the microtubules and regulate their stability [11]. Having observed role of LIMK1 in modulating microtubule stability, we performed Immunoprecipitation assay to examine if LIMK1 acts as MAP in PCa cells. Our data demonstrate that LIMK1 interacts with tubulin, as shown by the co-immunoprecipation of LIMK1 along with tubulin in PCa cells **(Figure 11)**. Interestingly, the amount of tubulin bound LIMK1 is decreased in CXCL12-stimulated PCa cells **(Figure 11)**. Together, our data support that LIMK1 acts as MAP, and CXCL12/CXCR4-signaling impedes DTX-induced microtubule stabilization by promoting the phosphorylation-mediated dissociation of LIMK1 from microtubules.

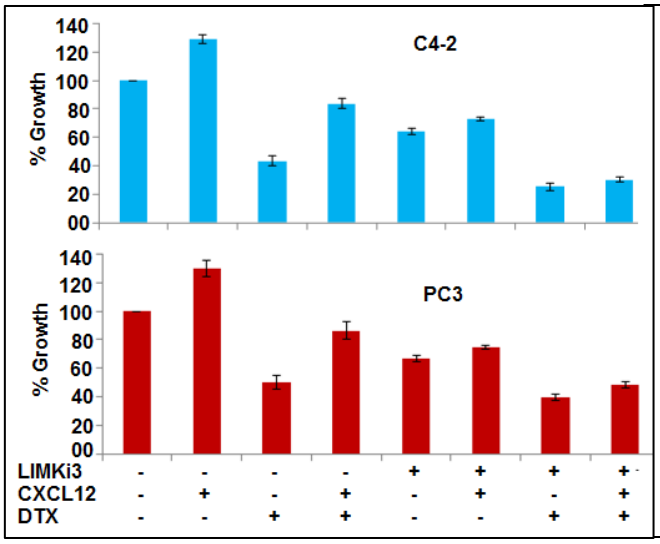

Figure 9: Inhibition of LIMK1 abrogates CXCL12-induced resistance to docetaxel toxicity. PCa cells were seeded in 96 well plate and treated with LIMKi3, CXCL12 and DTX alone or in combination as described earlier. After 48 h of treatment cell viability was measured by WST-1 assay. Data (mean ± SD; n=3) presented as change in cell viability as compared to the control cells.

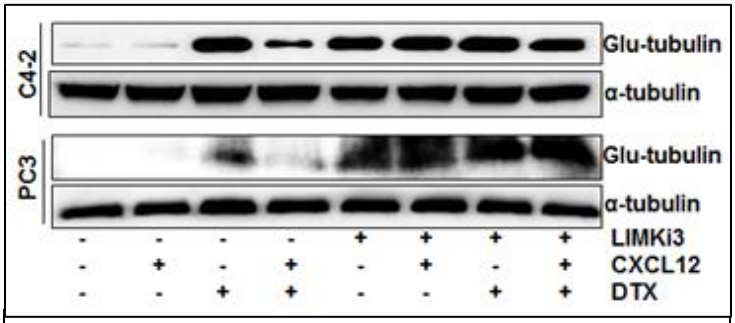

Figure 10: Effect of LIMK1 inhibition of the CXCL12-induced microtubule destabilization. C4-2 and PC3 cells grown in 6-well plate were treated with LIMKi3 (25 μM) for 2h, prior to the CXCL12 and DTX treatment alone or in combination. Post treatment total protein was collected and immunoblot analysis was performed to examine the expression of Gul- and Ace-tubulins. β-actin was used as an internal control.

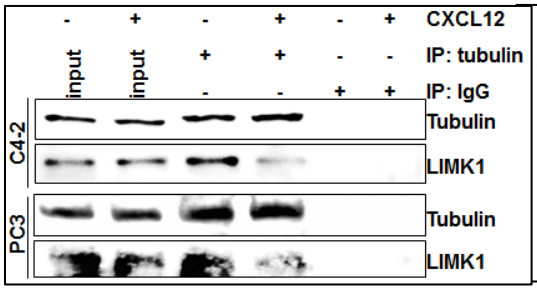

Figure 11: CXCL12-treatment decreases the association between tubulin and LIMK1. Immuno-precipitation assay with anti-tubulin (mouse mAb) or normal mouse IgG antibodies using the total cell lysates from control and with CXCL12-stimulated PCa cells was performed. Thereafter, proteins were resolved by electrophoresis and subjected to immunoblot analysis for tubulin and LIMK1.

CXCL12/CXCR4-induced activation of LIMK1 is mediated through PAK4. Further to delineate the molecular mechanism of CXCL12 induced LIMK1 activation, we focused on PAK4,

an upstream activator kinase of LIMK1 [12] and overexpressed in PCa [13]. As depicted in **Figure 12A** CXCL12 activates the PAK4 early time point (5 min) than the LIMK1 (5 min), suggesting that PAK4 activation is an early event than LIMK1 activation. Interestingly, this effect was reversed upon inhibition of CXCR4 **(Figure 12B)**. Next, we silenced PAK4 using specific siRNAs prior to the CXCL12 stimulation and effect on the p-LIMK1 was examined. Data demonstrate that effect of CXCL12 on the LIMK1 phosphorylation is abrogated in PAK4 silenced PCa cells, which strongly suggest the involvement of PAK4 in the CXCL12-mediated phosphorylation of LIMK1 **(Figure 13)**.

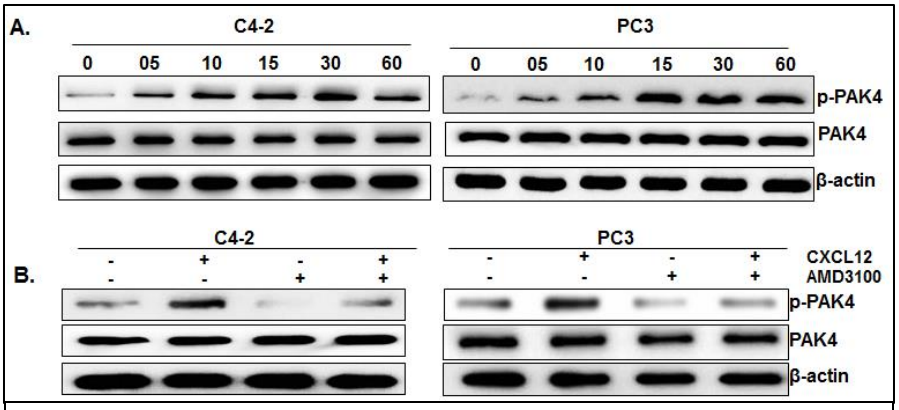

Figure 12: CXCL12/CXCR4 enhances the phosphorylation of PAK4. A. C4-2 and PC3 cells were grown in 6-well plate and treated with CXCL12 (100 ng/mL) for various time intervals (0-120 min). Thereafter, total protein was isolated, resolved, and subjected to immunoblot analysis to determine the expression of p-PAK4 and total-PAK4. **B.** Cells were pretreated with AMD3100 (5μM) 1h prior to the CXCL12 treatment (100 ng/mL). After 60 min, effect on the expression of p-PAK4 and total-PAK4 was examined by immunoblot assay.

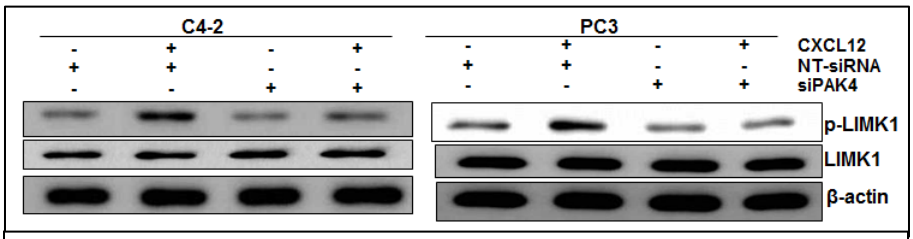

Figure 13: PAK4-mediates CXCL12/CXCR4-induced LIMK1 phosphorylation. C4-2 and PC3 cells were transfected with PAK4 targeting or non-targeting siRNAs (NT-siRNA). After 24 h of transfection cells were treated with CXCL12 for 60 min. Post treatment, phosphorylation status of LIMK1 was determined by immunoblot assay.

KEY RESEARCH ACCOMPLISHMENTS

- We have provided experimental evidence for the chemoprotective role of CXCL12/CXCR4 signaling in docetaxel-induced cytotoxicity in PCa cells.

- We have explored the mechanistic insight of the CXCL12/CXCR4-induced docetaxel resistance in prostate cancer cells. Our data indicate that CXCL12/CXCR4 rescue prostate cancer cells from DTX-induced cytotoxicity by counteracting DTX-induced microtubules polymerization and subsequent G2/M cell cycle arrest in PCa cells.

- We demonstrated that inhibition of LIMK1 abrogates CXCL12-induced resistance to docetaxel toxicity and microtubule destabilization.

- Finally, our data show that CXCL12/CXCR4-induced activation of LIMK1 is mediated through PAK4.

CONCLUSION

Our research findings demonstrate the role of CXCL12/CXCR4 signaling axis in DTX-resistance of prostate cancer cells. Therefore, targeting of this signaling node could be useful for therapeutic enhancement of DTX.

REPORTABLE OUTCOME

Poster presentation:
We presented a poster entitled “A novel CXCR4-mediated mechanism of docetaxel resistance in prostate cancer cells” by Bhardwaj A, Srivastava SK, Arora A, Hyde SJ, Andrews JF, McClellan S, Singh S, and Singh AP, in 104th Annual Meeting of American Association for Cancer Research (AACR), held at Washington, DC, April 6-10, 2013.

Manuscript:
Bhardwaj A, Srivastava SK, Singh S, Arora A, Hyde SJ, Andrews JF, McClellan S, and Singh AP. CXCL12/CXCR4 signaling counteracts docetaxel-induced microtubule stabilization via p21-activated kinase 4- dependent activation of LIM domain kinase 1. **J. Biol. Chem.** (near submission)

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APPENDICES:

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Poster Presentations - Cancer Mechanisms and Medicine 2

Abstract 4061: A novel CXCR4-mediated mechanism of docetaxel resistance in prostate cancer cells.

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Docetaxel (DTX), a semi-synthetic analog of paclitaxel, has emerged as the standard of care for chemotherapy of hormone-resistant prostate cancer. DTX confers its anti-neoplastic activity by inhibiting microtubule depolymerization, which leads to G2/M cell cycle arrest and subsequent apoptosis. However, most patients treated with DTX ultimately develop resistance to the drug and succumb to the disease. Therefore, understanding the mechanisms underlying DTX resistance is a priority area in prostate cancer research. Increasingly, it is being suggested that cancer cells may use common pathways for disease aggressiveness/metastatic spread and chemotherapeutic resistance. Therefore, we investigated the role of CXCL12/CXCR4 signaling, which is known to promote invasion and metastasis, in DTX-resistance of prostate cancer cells. Our data demonstrated that CXCL12 treatment rescued the prostate cancer cells from DTX-induced cytotoxicity and G2/M mitotic arrest. Furthermore, the cytoprotective effect of CXCL12 was abolished upon pretreatment of prostate cancer cells with AMD3100 (a small molecule antagonist of CXCR4) or siRNA-mediated silencing of CXCR4, thus confirming the role of CXCR4 in DTX-resistance. Immunofluorescence analyses revealed enhanced polymerization of microtubules in DTX-treated prostate cancer cells. In accordance with this, immunoblot analyses demonstrated increased levels of detyrosinated (glu-) and acetylated (ace-) tubulins, specific markers of the polymerized tubulin, in prostate cancer cells treated with DTX. Co-treatment of CXCL12 abrogated DTX-induced stabilization of microtubules in the prostate cancer cells, an effect that was diminished when the cells were pre-treated with AMD3100. Altogether, these initial findings demonstrate the role of CXCL12/CXCR4 signaling axis in DTX-resistance of prostate cancer cells and thus could be an attractive target for therapeutic enhancement of DTX.

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